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Enclosed is a copy of Priority Document 98202465.5 filed July 22, 1998 for the above referenced application.

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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

98202465.5

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(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
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Streptococcus vaccines comprising capsular deficient mutants

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Title: Streptococcus vaccines comprising capsular deficient mutants.

The invention relates to the field of bacterial vaccines, more in particular to vaccines directed against *Streptococcus* infections.

Streptococcus species, of which there are a large variety causing infections in domestic animals and man, are often grouped according to Lancefield's groups. Typing according to Lancefield occurs on the basis of serological determinants or antigens that are among others present in the capsule of the bacterium and allows for only an approximate determination, often bacteria from a different group show cross-reactivity with each other, while other Streptococci can not be assigned a group-determinant at all. Within groups, further differentiation is often possible on the basis of serotyping; these serotypes further contribute to the large antigenic variability of Streptococci, a fact that creates an array of difficulties within diagnosis of and vaccination against Streptococcal infections.

Lancefield group A *Streptococcus species* (GAS, *Streptococcus pyogenes*), are common with children, causing nasopharyngeal infections and complications thereof. Among animals, especially cattle are susceptible to GAS, whereby often mastitis is found.

Group A streptococci are the etiologic agents of streptococcal pharyngitis and impetigo, two of the commonest bacterial infections in children, as well as a variety of less common but potentially life-threatening infections, including soft tissue infections, bacteraemia, and pneumonia. In addition, GAS are uniquely associated with the postinfectious autoimmune syndromes of acute rheumatic fever and poststreptococcal glomerulonephritis.

Several recent reports suggest that the incidence both of serious infections due to GAS and of acute rheumatic fever has

increased during the past decade, focusing renewed interest on defining the attributes or virulence factors of the organism that may play a role in the pathogenesis of these diseases.

GAS produce several surface components and extracellular products that may be important in virulence. The major surface protein, M protein, has been studied in the most detail and has been shown convincingly to play a role in both virulence and immunity. Isolates rich in M protein are able to grow in human blood, a property thought to reflect the capacity of M protein to interfere with phagocytosis, and these isolates tend to be virulent in experimental animals.

Clinical observations have suggested that the hyaluronic acid capsule is also important in virulence: mucoid, or highly encapsulated, strains are uncommon among clinical isolates of GAS in general, but appear to be proportionally more frequent among GAS isolates associated with invasive infections or acute rheumatic fever. In a survey of more than 1,100 GAS isolates collected in the United States between 1988 and 1990, Johnson et al. found that only 3% of pharyngitis isolates were mucoid. In contrast, 21% of the strains associated with invasive infections were mucoid, and 43% of the isolates associated with acute rheumatic fever were mucoid. Although some bias in the collection of these strains cannot be excluded, the strikingly increased prevalence of mucoidy among invasive and rheumatic fever-associated isolates suggests an association between capsule expression and virulence.

Lancefield group B *Streptococcus* (GBS) are most often seen with cattle, causing mastitis, however, human infants are susceptible as well, often with fatal consequences. Group B streptococci (GBS) constitute a major cause of bacterial sepsis and meningitis among human neonates born in the United States and Western Europe and are emerging as significant neonatal pathogens in developing countries as well.

It is estimated that GBS strains are responsible for 10,000 to 15,000 cases of invasive infection in neonates in the United States alone. Despite advances in early diagnosis

and treatment, neonatal sepsis due to GBS continues to carry a mortality rate of 15 to 20%. In addition, survivors of GBS meningitis have 30 to 50% incidence of long-term neurologic sequelae. The increasing recognition over the past two decades of GBS as an important pathogen for human infants has generated renewed interest in defining the bacterial and host factors important in virulence of GBS and in the immune response to GBS infection.

Particular attention has focused on the capsular polysaccharide as the predominant surface antigen of the organisms. In a modification of the system originally developed by Rebecca Lancefield, GBS strains are serotyped on the basis of antigen differences in their capsular polysaccharides and the presence or absence of serologically defined C proteins. While GBS isolated from non-human sources often lack a serologically detectable capsule, a large majority of strains associated with neonatal infection belong to one of four major capsular serotypes, 1a, 1b, II or III. The capsular polysaccharide forms the outermost layer around the exterior of the bacterial cell, superficial to the cell wall. The capsule is distinct from the cell wall-associated group B carbohydrate.

The group B polysaccharide, in contrast to the type-specific capsule, is present on all GBS strains and is the basis for serogrouping of the organisms into Lancefield's group B. Early studies by Lancefield and co-workers showed that antibodies raised in rabbits against whole GBS organisms protected mice against challenge with strains of homologous capsular type, demonstrating the central role of the capsular polysaccharide as a protective antigen. Studies in the 1970s by Baker and Kasper demonstrated that cord blood of human infants with type III GBS sepsis uniformly had low or undetectable levels of antibodies directed against the type III capsule, suggesting that a deficiency of anticapsular antibody was a key factor in susceptibility of human neonates to GBS disease.

Lancefield group C infections, such as those with *S. equi, S. zooepidemicus, S. dysgalactiae*, and others are mainly seen with horse, cattle and pigs, but can also cross the species barrier to humans. Lancefield group D (*S. bovis*) infections are found with all mammals and some birds, sometimes resulting in endocarditis or septicaemia.

Lancefield groups E, G, L, P, U and V (*S. porcinus, S, canis, S. dysgalactiae*) are found with various hosts, causing neonatal infections, nasopharyngeal infections or mastitis.

Within Lancefield groups R, S, and T, (and with ungrouped types) *S. suis* is found, an important cause of meningitis, septicemia, arthritis and sudden death in young pigs. Incidentally, it can also cause meningitis in man.

S. suis strains are identified by their morphological, biochemical and serological characteristics. Serological classification is based on the presence of specific antigenic polysaccharides. So far, 35 different capsular types have been described. In several European countries, *S. suis* serotype 2 is the most prevalent type isolated from diseased pigs, followed by serotypes 1 and 9.

Little is known about the pathogenesis of the disease caused by *S. suis* type 2. Various cellular components, such as muramidase-released protein (MRP) extracellular factor (EF) and cell-membrane associated proteins, fimbriae, haemagglutinins, and haemolysin have been suggested as virulence factors. However, the precise role of these protein components in the pathogenesis of the disease remains unclear.

It is however, well known and generally accepted that the polysaccharidic capsule of various Streptococci and other gram-positive bacteria plays an important role in pathogenesis. The capsule enables these micro-organisms to resist phagocytosis and is therefore regarded as an important virulence factor.

Compared to encapsulated *S. suis* strains, non-encapsulated *S. suis* strains are phagocytosed by murine polymorphonuclear leucocytes to a greater degree. Moreover, an

increase in thickness of capsule was noted for *in vivo* grown virulent strains while no increase was observed for avirulent strains. Therefor, these data again demonstrate the role of the capsule in the pathogenesis for *S. suis* as well.

Ungrouped *Streptoccus species*, such as *S. mutans*, causing carries with humans, *S, uberis*, causing mastitis with cattle, and *S. pneumonia*, causing major infections in humans, and *Enterococcus faecilalis* and *E. faecium*, further contributed to the large group of Streptococci.

Streptococcus pneumoniae (the pneumococcus) is a human pathogen causing invasive diseases, such as pneumonia, bacteraemia, and meningitis. Despite the availability of antibiotics, pneumococcal infections remain common and can still be fatal, especially in high-risk groups, such as young children and elderly people. Particularly in developing countries, many children under the age of five years die each year from pneumococcal pneumonia. S. *pneumoniae* is also the leading cause of otitis media and sinusitis. These infections are less serious, but nevertheless incur substantial medical costs, especially when leading to complications, such as permanent deafness. The normal ecological niche of the pneumococcus is the nasopharynx of man. The entire human population is colonised by the pneumococcus at one time or another, and at a given time, up to 60% of individuals may be carriers. Nasopharyngeal carriage of pneumococci by man is often accompanied by the development of protection to infection by the same serotype. Most infections do not occur after prolonged carriage but follow the acquisition of recently acquired strains. Many bacteria contain surface polysaccharides which act as a protective layer against the environment. Surface polysaccharides of pathogenic bacteria usually make the bacteria resistant to the defense mechanisms of the host, e.g., the lytic action of serum or phagocytosis. In this respect, the serotype-specific capsular polysaccharide (CP) of *Streptococcus pneumoniae*, is an important virulence factor. Unencapsulated strains are avirulent, and antibodies

directed against the CP are protective. Protection is serotype specific; each serotype has its own, specific CP structure. Ninety different capsular serotypes have been identified. Currently, CPs of 23 serotypes are included in a vaccine.

Vaccines directed against *Streptococcus* infections in general aim at utilising an immune response directed against the polysaccharide capsule of the various *Streptococcus species*, especially since the capsule is considered a main virulence factor for these bacteria. The capsule, during infection, provides resistance to phagocytosis and thus promotes the escape of the bacteria from the immune system of the host, protecting the bacteria by elimination by macrophages and neutrophils.

The capsule particularly confers the bacterium resistance to complement-mediated opsonophagocytosis. In addition, some bacteria express capsular polysaccharides (CPs) that mimic host molecules, thereby avoiding the immune system of the host. Also, even when the bacteria have been phagocytosed, intracellular killing is hampered by the presence of a capsule.

It is in general thought that only when the host has antibodies or other serum-factors directed against capsule antigens, the bacterium will get recognised by the immune system through the anticapsular-antibodies or serum-factors bound to its capsule, and will, through opsonisation, get phagocytosed and killed.

However, these antibodies are serotype-specific, and will often only confer protection against only one of the many serotypes known within a group of *Streptococci*.

For example, current commercially available *S. suis* vaccines, which are in general based on whole-cell-bacterial preparations, or on capsule-enriched fractions of *S. suis*, confer only limited protection against heterologous strains. Also, the current pneumococcal vaccine, licensed in the United States in 1983, consists of purified CPs of 23 pneumococcal serotypes whereas at least 90 CP types exist.

The composition of this pneumococcal vaccine was based on the frequency of the occurrence of disease isolates in the US and cross-reactivity between various serotypes. Although this vaccine protects healthy adults against infections caused by serotypes included in the vaccine, it fails to raise a protective immune response in infants younger than 18 months and it is less effective in elderly people. In addition, the vaccine confers only limited protection in patients with immunodeficiencies and haematology malignancies.

In the light of above, improved vaccines are needed against *Streptococcus* infections. Much attention is being paid at producing CP vaccines by producing the relevant polysaccharides via chemical or recombinant means. However, chemical synthesis of polysaccharides is costly, and capsular polysaccharide synthesis by recombinant means necessitates knowledge about the relevant genes, which are not always available and need to de determined for each and every relevant serotype.

The invention provides an acapsular *Streptococcus* mutant for use in a vaccine, a vaccine strain derived thereof and a vaccine derived thereof. Surprisingly, and against the grain of common doctrine, the invention does not focus on using (purified or isolated) capsular *Streptococcus* antigens or whole-cell-preparations comprising capsular *Streptococcus* antigens for a vaccine but provides use of a *Streptococcus* mutant deficient in capsular expression in a vaccine.

Acapsular *Streptococcus* mutants have long been known in the art and can be found in nature. Griffith (J. Hyg. 27:113-159, 1928) demonstrated that pneumococci could be transformed from one type to another. If he injected live rough (acapsular or unencapsulated) type 2 pneumococci into mice, the mice would survive. If, however, he injected the same dose of live rough type 2 mixed with heat-killed smooth (encapsulated) type 1 into a mouse, the mouse would die, and from the blood he could isolate live smooth type 1 pneumococci. At that time,

the significance of this transforming principle was not understood. However, understanding came when it was shown that DNA constituted the genetic material responsible for phenotypic changes during transformation.

Streptococcus mutants deficient in capsular expression are found in several forms. Some are fully deficient and have no capsule at all, others form a deficient capsule, characterised by a mutation in a capsular gene cluster. Deficiency can for instance include capsular formation wherein the organization of the capsular material has been re-arranged, as for example demnosrable by electron microscopy. Yet others have a nearly fully developed capsule which is only deficient in a particular sugar component.

Now, after much advance of biotechnology and despite the fact that little is still known about the exact localisation and sequence of genes involved in capsular synthesis in Streptococci, it is possible to create mutants of Streptococci, for example by homologous recombination or transposon mutagenesis, which has for example been done for GAS (Wessels et al., PNAS 88:8317-8321, 1991), for GBS (Wesels et al., PNAS 86: 8983-8987, 1989), for *S. suis* (Smith, ID-DLO Annual report 1996, page 18-19; Charland et al., Microbiol. 144:325-332, 1998) and for *S. pneumonia* (Kolkman et al., J. Bact. 178:3736-3741, 1996). Such recombinant derived mutants, or isogenic mutants, can easily be compared with the wild-type strains from which they have been derived.

In a preferred embodiment, the invention provides use of a recombinant-derived *Streptococcus* mutant deficient in capsular expression in a vaccine. Recombinant techniques useful in producing such mutants are for example homologous recombination, transposon mutagenises, and others, whereby deletions, insertions or (point)-mutations are introduced in the genome. Advantages of using recombinant techniques are the stability of the obtained mutants (especially with homologous recombination and double cross-over techniques), and the

knowledge about the exact site of the deletion, mutation or insertion.

In a much preferred embodiment, the invention provides a stable mutant deficient in capsular expression obtainable for example through homologous recombination or cross over integration events. Examples of such a mutant can be found in the experimental part of this description, for example mutant 10cpsB or 10cpsEF is such a stable mutant as provided by the invention.

The invention also provides a *Streptococcus* vaccine strain and vaccine that has been derived from a *Streptococcus* mutant deficient in capsular expression. In general, said strain or vaccine is applicable within the whole range of Streptococcal infections, be it for those with animals or man or with zoonotic infections. It is of course now possible to first select a common vaccine strain and derive a *Streptococcus* mutant deficient in capsular expression thereof for the selection of a vaccine strain and use in a vaccine according to the invention.

In a preferred embodiment, the invention provides use of a *Streptococcus* mutant deficient in capsular expression in a vaccine wherein said *Streptococcus* mutant is selected from the group composed of *Streptococcus* group A, *Streptococcus* group B, *Streptococcus suis* and *Streptococcus pneumonia*. Herewith the invention provides vaccine strains and vaccines for use with these notoriously heterologous Streptococci, of which a multitude of serotypes exist. With a vaccine as provided by the invention that is derived from a specific *Streptococcus* mutant that deficient in capsular expression, the difficulties relating to lack of heterologous protection can be circumvented since these mutants do nor rely on capsular antigens per se to induce protection.

In a preferred embodiment, said vaccine strain is selected for its ability to survive or even replicate in an immune-competent host or host cells and thus can persist for a

certain period, varying from 1-2 days to more than one or two weeks, in a host, despite its deficient character.

Although an immunodeficient host will support replication of a wide range of bacteria that are deficient in one or more virulence factors, in general it is considered a characteristic of pathogenicity of Streptococci that they can survive for certain periods or replicate in a normal host or host cells such as macrophages. For example, Wiliams and Blakemore (Neuropath. Appl. Neurobiol.: 16, 345-356, 1990; Neuropath. Appl. Neurobiol.: 16, 377-392, 1990; J. Infect. Dis.: 162, 474-481, 1990) show that both polymorphonuclear cells and macrophage cells are capable of phagocytosing pathogenic *S. suis* in pigs lacking anti-*S. suis* antibodies, only pathogenic bacteria could survive and multiply inside macrophages and the pig.

In a preferred embodiment, the invention, however, provides a deficient or avirulent mutant or vaccine strain which is capable of surviving at least 4-5 days, preferably at least 8-10 days in said host, thereby allowing the development of a solid immune response to subsequent *Streptococcus* infection,

Due to its persistent but avirulent character, a *Streptococcus* mutant or vaccine strain as provided by the invention is well suited to generate specific and/or long-lasting immune responses against Streptococcal antigens, moreover because possible specific immune responses of the host directed against a capsule are relatively irrelevant because a vaccine strain as provided by the invention is in general not recognised by such antibodies.

In addition, the invention provides a *Streptococcus* vaccine strain according the invention which strain comprises a mutant capable of expressing a *Streptococcus* virulence factor or antigenic determinant.

In a preferred embodiment, the invention provides a *Streptococcus* vaccine strain according to the invention which strain comprises a mutant capable of expressing a

Streptococcus virulence factor wherein said virulence factor or antigenic determinant is selected from a group of cellular components, such as muramidase-released protein (MRP) extracellular factor (EF) and cell-membrane associated proteins, 60kDA heat shock protein, pneumococcal surface protein A (Psp A), pneumolysin, C protein, protein M, fimbriae, haemagglutinins and haemolysin or components functionally related thereto.

In a much preferred embodiment, the invention provides a *Streptococcus* vaccine strain according to the invention which strain comprises a mutant capable of over-expressing said virulence factor. In this way, the invention provides a vaccine strain for incorporation in a vaccine which specifically causes a host to provide a immune response directed against antigenically important determinants of virulence (listed above), thereby providing specific protection directed against said determinants. Over-expression can for example be achieved by cloning the gene involved behind a strong promoter, which is for example constitutionally expressed in a multicopy system, either in a plsamid or via intergration in a genome.

In yet another embodiment, the invention provides a *Streptococcus* vaccine strain according to the invention which comprises a mutant capable of expressing a non-*Streptococcus* protein. Such a vector-*Streptococcus* vaccine strain allows, when used in a vaccine, protection against other pathogens than Streptococcus.

Due to its persistent but avirulent character, a *Streptococcus* vaccine strain or mutant as provided by the invention is well suited to generate specific and long-lasting immune responses, not only against Streptococcal antigens, but also against other antigens when these are expressed by said strain. Especially antigens derived from another pathogen are now expressed without the detrimental effects of said antigen or pathogen which would otherwise have harmed the host.

An example of such a vector is a *Streptococcus* vaccine strain or mutant wherein said antigen is derived from a pathogen, such as *Actinobacillus pleuropneumonia, Mycoplasmatae, Bordetella, Pasteurella, E. coli, Salmonella, Campylobacter, Serpulina* and others.

The invention also provides a vaccine comprising a *Streptococcus* vaccine strain or mutant according to the invention and further comprising a pharmaceutically acceptable carrier or adjuvant. Carriers or adjuvants are well known in the art, examples are phosphate buffered saline, physiological salt solutions, (double-)oil-in-water-emulsions, aluminumhydroxide, Specol, block- or co-polymers, and others.

A vaccine according to the invention can comprise a vaccine strain either in a killed or live form. For example, a killed vaccine comprising a strain having (over)expressed a Streptococcal or heterologous antigen or virulence factor is very well suited for eliciting an immune response. In a preferred embodiment, the invention provides a vaccine wherein said strain is live, due to its persistent but avirulent character, a *Streptococcus* vaccine strain as provided by the invention is well suited to generate specific and long-lasting immune responses.

Now that a Streptococcal vaccine is provided by the invention, the invention also provides a method for controlling or eradicating a Streptococcal disease in a population comprising vaccinating subjects in said population with a vaccine according to the invention.

In a preferred embodiment, a method for controlling or eradicating a Streptococcal disease is provided comprising testing a sample, such as a blood sample, or nasal or throat swab, faeces, urine, or other samples such as can be sampled at or after slaughter, collected from at least one subject, such as an infant or a pig, in a population partly or wholy vaccinated with a vaccine according to the invention for the presence of encapsulated Streptococcal strains or mutants. Since a vaccine strain or mutant according to the invention is

not pathogenic, and can be distinguished from wild-type strains by capsular expression, the detection of (fully) encapsulated Streptococcal strains indicates that wild-type infections are still present. Such wild-type infected subjects can than be isolated from the remainder of the population until the infection has passed away. With domestic animals, such as pigs, it is even possible to remove the infected subject from the population as a whole by culling. Detection of wild-type strains can be achieved via traditional culturing techniques, or by rapid detection techniques such as PCR detection.

In yet another embodiment, the invention provides a method for controlling or eradicating a Streptococcal disease comprising testing a sample collected from at least one subject in a population partly or wholly vaccinated with a vaccine according to the invention for the presence of capsule-specific antibodies directed against Streptococcal strains. Capsule specific antibodies can be detected with classical techniques known in the art, such as used for Lancefield's group typing or serotyping.

A much preferred embodiment of a method provided by the invention for controlling or eradicating a Streptococcal disease in a population comprises vaccinating subjects in said population with a vaccine according to the invention and testing a sample collected from at least one subject in said population for the presence of encapsulated Streptococcal strains and/or for the presence of capsule-specific antibodies directed against Streptococcal strains.

For example, a method is provided according to the invention wherein said Streptococcal disease is caused by *Streptococcus suis*.

The invention also provides a diagnostic assay for testing a sample for use in a method according to the invention comprising at least one means for the detection of encapsulated Streptococcal strains and/or for the detection of

capsule-specific antibodies directed against Streptococcal strains.

The invention is further explained in the experimental part of the description without limiting the invention thereto.

Experimental part

Streptococcus suis is an important cause of meningitis, septicemia, arthritis and sudden death in young pigs (4, 46). Incidentally, it can also cause meningitis in man (1). *S. suis* strains are identified by their morphological, biochemical and serological characteristics. Serological classification is based on the presence of specific antigenic polysaccharides. So far, 35 different serotypes have been described (9, 14). In several European countries, *S. suis* serotype 2 is the most prevalent type isolated from diseased pigs, followed by serotypes 9 and 1. Little is known about the pathogenesis of the disease caused by *S. suis* type 2. Various bacterial components, such as extracellular and cell-membrane associated proteins, fimbriae, haemaglutinins, and haemolysin have been suggested as virulence factors (9, 10, 11, 15, 16, 47, 49). However, the precise role of these protein components in the pathogenesis of the disease remains unclear (37).

It is well known that the polysaccharidic capsule of various Streptococci and other gram-positive bacteria plays an important role in pathogenesis (3, 6, 35, 51, 52). The capsule enables these micro-organisms to resist phagocytosis and is therefore regarded as an important virulence factor. Recently, a role of the capsule of *S. suis* in the pathogenesis was suggested as well (5). Within *S. suis* serotypes 1 and 2 strains can differ in virulence for pigs (41, 45, 49). Some type 1 and 2 strains are virulent, other strains are not. Because both virulent and non-virulent strains of serotype 1 and 2 strains are fully encapsulated, these data clearly show that capsule is

not the only factor required for virulence. Therefore, to provide conclusive data regarding the role of the capsule in the pathogenesis isogenic mutants impaired in capsule production are required.

Biosynthesis of capsule polysaccharides has been studied in a number of Gram-positive and Gram-negative bacteria (32). In Gram-negative bacteria, but also in a number of gram-positive bacteria, the genes which are involved in the biosynthesis of polysaccharides are clustered at a single locus (32). Moreover, the capsular genes showed a common genetic organisation involving three distinct regions (32). The central region is type specific and encodes enzymes responsible for the synthesis and polymerisation of the polysaccharides. This region is flanked by two conserved regions presumed to encode proteins for common functions such as transport of the polysaccharide across the cellular membrane.

The structure, organisation and functioning of the genes responsible for capsule polysaccharide synthesis (*cps*) in *S. suis* is unknown. In the present paper we describe the isolation and molecular characterisation of a 16 kb fragment containing the 5' end of the *cps* gene cluster of *S. suis* type 2. In addition, we show that isogenic mutants obtained after double cross-over recombination events in the *cps2B* or *cps2EF* genes are resistant to phagocytosis by alveolar lung macrophages *in vitro* and are not virulent for young germfree pigs. Attempts to control *S. suis infections* or disease are still hampered by the lack of knowledge about the epidemiology of the disease and the lack of effective vaccines and sensitive diagnostics.

S. suis strains are usually identified and classified by their morphological, biochemical and serological characteristics (58, 59, 46). Serological classification is based on the presence of specific antigenic polysaccharides. So far, 35 different serotypes have been described (9, 56, 14). In several European countries, *S. suis* serotype 2 is the most prevalent type isolated from diseased pigs, followed by

serotypes 9 and 1. Serological typing of *S. suis* is carried out using different types of agglutination tests. In these tests, isolated and biochemically characterized *S. suis* cells are agglutinated with a panel of 35 specific sera. These methods are very laborious and time-consuming. A comparison of the predicted amino acid sequences of the *cps2* genes with sequences found in the databases allowed the tentative assignment of functions to most of the open reading frames. The organization of the *cps* 2 locus of *S. suis* type 2 seemed to be identical to the organization of the *cps* loci in a number of Gram-negative and Gram-positive bacteria (19, 32, 63). The central region seemed to contain the type specific glycosyltransferases and the putative polysaccharide polymerase. This region is flanked by two regions presumed to encode for proteins with common functions, such as regulation and transport of polysaccharide across the membrane..

We further describe the isolation and molecular characterization of the type specific *cps* genes of *S. suis* types 1 and 9. In addition, we describe the genetic diversity of the *cps* loci of serotypes 1, 2 and 9 among the 35 *S. suis* serotypes. Type-specific probes could be identified. Based on these data we developed a type-specific PCR for serotype 9. The PCR is a rapid, reliable and sensitive assay. Moreover, we showed that this PCR could be used directly on nasal or tonsillar swabs of infected or carrier animals.

MATERIAL AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* strains were grown in Todd-Hewitt broth (code CM189, Oxoid), and plated on Columbia agar blood base (code CM331, Oxoid) containing 6% (v/v) horse blood. *E.coli* strains were grown in Luria broth (28) and plated on Luria broth containing 1.5% (w/v) agar. If required, antibiotics were added to the plates at the following

concentrations: spectinomycin: 100 ug/ml for *S. suis* and 50 ug/ml for *E. coli* and ampicillin, 50 ug/ml.

Serotyping. The *S.suis* strains were serotypes by the slide agglutination test with serotype-specific antibodies (44).

DNA techniques. Routine DNA manipulations were performed as described by Sambrook et al. (36).

Alkaline phosphatase activity. To screen for PhoA fusions in *E.coli*, plasmid libraries were constructed. Therefore, chromosomal DNA of *S. suis* type 2 was digested with *Alu*I. The 300-500-bp fragments were ligated to *Sma*I-digested pPHOS2. Ligation mixtures were transformed to the PhoA$^-$ *E. coli* strain CC118. Transformants were plated on LB media supplemented with 5-Bromo-4-chloro-3-indolylfosfaat (BCIP, 50 ug/ml, Boehringer, Mannheim, Germany). Blue colonies were purified on fresh LB/BCIP plates to verify the blue phenotype.

DNA sequence analysis. DNA sequences were determined on a 373A DNA Sequencing System (Applied Biosystems, Warrington, GB). Samples were prepared by use of a ABI/PRISM dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequencing data were assembled and analyzed using the MacMollyTetra program. Custom-made sequencing primers were purchased from Life Technologies. Hydrophobic stretches within proteins were predicted by the method of Klein et al. (17). The BLAST program available on Netscape Navigator^{TM} was used to search for protein sequences homologous to the deduced amino acid sequences.

Construction of gene-specific knock-out mutants of *S. suis*. To construct the mutant strains 10cpsB and 10cpsEF we electrotransformed the pathogenic serotype 2 strain 10 (45, 49) of *S. suis* with pCPS11 and pCPS28 respectively. In these plasmids the *cps*B and *cps*EF genes were disturbed by the insertion of a spectinomycin-resistance gene. To create pCPS11 the internal 400 bp *Pst*I-*Bam*HI fragment of the *cps*B gene in pCPS7 was replaced by the Spc^R gene. For this purpose pCPS7 was digested with *Pst*I and *Bam*HI and ligated to the 1,200-bp *Pst*I-

*Bam*HI fragment, containing the Spc^R gen, from pIC-spc. To construct pCPS28 we have used pIC20R. In this plasmid we inserted the *Kpn*I-*Sal*I fragment from pCPS17 (resulting in pCPS25) and the *Xba*I-*Cla*I fragment from pCPS20 (resulting in pCPS27). pCPS27 was digested with *Pst*I and *Xho*I and ligated to the 1,200-bp *Pst*I-*Xho*I fragment, containing the Spc^R gene of pIC-spc. The electrotransformation to *S. suis* was carried out as described before (38).

Southern blotting and hybridization. Chromosomal DNA was isolated as described by Sambrook *et al.* (36). DNA fragments were separated on 0.8% agarose gels and transferred to Zeta-Probe GT membranes (Bio-Rad) as described by Sambrook *et al.* (36). DNA probes were labelled with [($-^{32}$P]dCTP (3000 Ci mmol^{-1}; Amersham) by use of a random primed labelling kit (Boehringer). The DNA on the blots was hybridized at 65°C with appropriate DNA probes as recommended by the supplier of the Zeta-Probe membranes. After hybridization, the membranes were washed twice with a solution of 40 mM sodium phosphate, pH 7.2, 1 mM EDTA , 5% SDS for 30 min at 65°C and twice with a solution of 40 mM sodium phosphate, pH 7.2, 1 mM EDTA, 1% SDS for 30 min at 65°C.

Electron Microscopy. Bacteria were prepared for electron microscopy as described by Wagenaar et al. (50). Shortly, bacteria were mixed with agarose MP (Boehringer) of 37° C to a concentration of 0.7%. The mixture was immediately cooled on ice. Upon gelifying, samples were cut into 1 to 1.5 mm slices and incubated in a fixative containing 0.8% glutaraldehyde and 0.8% osmiumtetraoxide. Subsequently, the samples were fixed and stained with uranyl acetate by microwave stimulation, dehydrated and imbedded in eponaraldite resin. Ultra-thin sections were counterstained with lead citrate and examined with a Philips CM 10 electron microscope at 80 kV.

Isolation of porcine alveolar macrophages (AM). Porcine AM were obtained from the lungs of specific pathogen free (SPF) pigs.

Lung lavage samples were collected as described by van Leengoed *et al.* (43). Cells were suspended in EMEM containing 6% (v/v) SPF-pig serum and adjusted to 10^7 cells per ml.

Phagocytosis assay. Phagocytosis assays were performed as described by Leij *et al.* (23). Briefly, to opsonize the cells, 10^7 *S. suis* cells were incubated with 6% SPF-pig serum for 30 min at 37°C in a head-over-head rotor at 6 rpm. 10^7 AM and 10^7 opsonized *S. suis* cells were combined and incubated at 37°C under continuous rotation at 6 rpm. At 0, 30, 60 and 90 min, 1-ml samples were collected and mixed with 4 ml of ice-cold EMEM to stop phagocytosis. Phagocytes were removed by centrifugation for 4 min at 110 x g and 4°C. The number of colony forming units (CFU) in the supernatants was determined. Control experiments were carried out simultaneously by combining 10^7 opsonized *S. suis* cells with EMEM (without AM).

Killing assays. The killing assay was described by Leij *et al.* (23). AM (10^7/ml) and opsonized *S. suis* cells (10^7/ml) were mixed 1 : 1 and incubated for 10 min at 37°C under continuous rotation at 6 rpm. Ice-cold EMEM was added to stop further phagocytosis and killing. To remove extracellular *S. suis* cells, phagocytes were washed twice (4 min, 110 x g, 4°C) and resuspended in 5 ml EMEM containing 6% SPF serum. The tubes were incubated at 37°C under rotation at 6 rpm. After 0, 15, 30, 60 and 90 min, samples were collected and mixed with ice-cold EMEM to stop further killing. The samples were centrifuged for 4 min at 110 x g at 4°C and the phagocytic cells were lysed in EMEM containing 1% saponine for 20 min at room temperature. The number of CFU in the suspensions was determined.

Pigs. Germfree pigs, cross-breeds of Great Yorkshire and Dutch landrace, were obtained from sows by caesarian sections. The surgery was performed in sterile flexible film isolators. Pigs were allotted to groups, each consisting of 4 pigs, and were housed in sterile stainless steel incubators. Housing

conditions and feeding regimes were as described before (45, 49).

Experimental infections. Pigs were inoculated intranasally with *S. suis* type 2 as described before (45, 49). To predispose the pigs for infection with *S. suis*, five-day old pigs were inoculated intranasally with about 10^7 CFU of *Bordetella bronchiseptica* strain 92932. Two days later the pigs were inoculated intranasally with *S. suis* type 2 (10^6 CFU). Pigs were monitored twice daily for clinical signs of disease, such as fever, nervous signs and lameness. Blood samples were collected three times a week from each pig. White blood cells were counted with a cell counter. To monitor infection with *S. suis* and *B. bronchiseptica* and to check for absence of contaminants, we collected swabs of nasopharynx and feces daily. The swabs were plated directly onto Columbia agar containing 6% horse blood. After three weeks the pigs were killed and examined for pathological changes. Tissue specimens from the central nervous system, serosae, and joints were examined bacteriologically and histologically as described before (45, 49). Colonization of the serosae was scored positively when *S. suis* was isolated from the pericardium, thoracal pleura or the peritoneum. Colonization of the joints was scored positively when *S. suis* was isolated from one or more joints (12 joints per animal were scored).

Vaccination and challenge

One week old pigs were vaccinated intravenously with a dosage of 106 cfu of the S. suis strains 10cpsEF or 10cpsB. Three weeks later the pigs were challenged intravenously with the pathogenic serotype 2 strain 10 (107 cfu). Disease monitoring, haematological, serological and bacteriological examinations as well as post-mortum examinations were as described before under experimental infections.

RESULTS

Identification of the *cps* locus.
The first part of the *cps* locus of *S.suis* type 2 was identified by making use of a strategy developed for the genetic identification of exported proteins (13, 31). In this system we made use of a plasmid (pPHOS2) containing a truncated alkaline phosphatase gene (13). The gene lacked the promoter sequence, the translational start site and the signal sequence. The truncated gene is precede by a unique *Sma*I restriction site. Chromosomal DNA of *S. suis* type 2, digested with *Alu*I, was randomly cloned in this restriction site. Because translocation of PhoA across the cytoplasmic membrane of *E. coli* is required for enzymatic activity, the system can be used to select for *S. suis* fragments containing a promoter sequence, a translational start site and a functional signal sequence. Among 560 individual *E. coli* clones tested, 16 displayed a dark blue phenotype when plated on media containing BCIP. DNA sequence analysis of the inserts from several of these plasmids were performed (results not shown) and the deduced amino acid sequences were analyzed. The hydrophobicity profile of one of the clones (pPHOS7, results not shown) showed that the N-terminal part of the sequence resembled the characteristics of a typical signal peptide: a short hydrophilic N-terminal region is followed by a hydrophobic region of 38 amino acids. These data indicate that the phoA system was successfully used for the selection of *S. suis* genes encoding exported proteins. Moreover, the sequences were analyzed for similarities present in the databases. The sequence of pPHOS7 showed a high similarity (37% identity) with the protein encoded by the *cps14C* gene of *Streptococcus pneumoniae* (19). This strongly suggests that pPHOS7 contains a part of the *cps* operon of *S. suis* type 2.

Cloning of the flanking *cps* genes. In order to clone the flanking *cps* genes of *S. suis* type 2 the insert of pPHOS7 was used as a probe to identify chromosomal DNA fragments which contain flanking *cps* genes. A 6-kb *Hind*III fragment was

identified and cloned in pKUN19. This yielded clone pCPS6 (Fig. 1C). Sequence analysis of the insert of pCPS6 revealed that pCPS6 most probably contained the 5'-end of the *cps* locus, but still lacked the 3'-end (see below). Therefore, sequences of the 3' -end of pCPS6 were in turn used as a probe to identify chromosomal fragments containing *cps* sequences located further downstream. These fragments were also cloned in pKUN19, resulting in pCPS17. Using the same system of chromosomal walking we subsequently generated the plasmid pCPS18, pCPS20, pCPS23 and pCPS26, containing downstream *cps* sequences.

Analysis of the cps operon. The complete nucleotide sequence of the cloned fragments was determined. Examination of the compiled sequence revealed the presence of at least 13 potential open reading frame (Orfs), which were designated as Orf 2Y, Orf2X and Cps2A-Cps2K (Fig. 1A). Moreover, a 14th, incomplete, Orf (Orf 2Z) was located at the 5'-end of the sequence. Two potential promoter sequences were identified. One was located 313 bp (locations 1885-1865 and 1884-1889) upstream of Orf2X. The other potential promoter sequence was located 68 bp upstream of Orf2Y (locations 2241-2236 and 2216-2211). Orf2Y is expressed in opposite orientation. Between Orfs 2Y and 2Z the sequence contained a potential stem-loop structure, which could act as a transcription terminator. Each Orf is preceded by a ribosome-binding site and the majority of the Orfs are very closely linked. The only significant intergenic gap was found between Cps2G and Cps2H (389 nucleotides). However, no obvious promoter sequences or potential stem-loop structures were found in this region. These data suggest that Orf2X and Cps2A-Cps2K are arranged as an operon.

An overview of all Orfs with their properties is shown in Table 2. The majority of the predicted gene products is homologous to proteins involved in polysaccharide biosynthesis. Orf2Z showed some similarity with the YitS protein of *Bacillus subtilis*. YitS was identified during the sequence analysis of

the complete genome of *B. subtilis*. The function of the protein is unknown.

Orf2Y showed homology with YcxD protein of *B. subtilis* (53). Based on the homology between YcxD and MocR of *Rhizobium meliloti* (33), YcxD was suggested to be a regulatory protein.

Orf2X showed homology with the hypothetical YAAA proteins of *Haemophilus influenzae* and *E. coli*. The function of these proteins is unknown.

The gene products encoded by the *cps2A*, *cps2B*, *cps2C* and *cps2D* genes showed significant homologies with the CpsA, CpsC, CpsD and CpsB proteins of several serotypes of *Streptococcus pneumoniae* (19), respectively. This strongly suggest similar functions for these proteins. Hence, Cps2A may have a role in the regulation of the capsular polysaccharide synthesis. Cps2B and Cps2C could be involved in the chain length determination of the type 2 capsule and Cps2C can play an additional role in the export of the polysaccharide. The Cps2D protein of *S. suis* is homologous to the CpsB protein of *S. pneumoniae* and to proteins encoded by genes of several other Gram-positive bacteria involved in polysaccharide or exopolysaccharide synthesis, but their function is unknown (19).

The protein encoded by *cps2E* gene showed homology to several bacterial proteins with glycosyl transferase activities: Cps14E and Cps19fE of *S. pneumoniae* serotypes 14 and 19F (18, 19, 29), CpsE of *Streptococcus salvarius* (X94980) and CpsD of *Streptococcus agalactiae* (34). Recently, Kolkman *et al.* (18) showed that Cps14E is a glucosyl-1-phosphate transferase that links glucose to a lipid carrier, the first step in the biosynthesis of the S. *pneumoniae* type 14 repeating unit. Based on these data a similar function may be fulfilled by Cps2E of *S. suis* .

The protein encoded by the *cps2F* gene showed similarity to the protein encoded by the *rfbU* gene of *Salmonella enteritica*. (25). This similarity is most pronounced in the C-terminal regions of these proteins. The *rfbU* gene was shown to encoded mannosyltransferase activity (25).

The *cps2G* gene encoded a protein that showed moderate similarity with the *rfbF* gene product of *Campylobacter hyoilei* (22), the *epsF* gene product of *S. thermophilus* (40) and the *capM* gene product of *S. aureus* (24). On the basis of homology the *rfbF, epsF* and *capM* genes are suggested to encoded galactosyltransferase activities. Hence, a similar glycosyl transferase activity could be fulfilled by the *cps2G* gene product.

The *cps2H* gene encodes a protein that is similar to the N-terminal region of the *lgtD* gene product of *Haemophilus influenzae* (U32768). Moreover, the hydrophobicity plots of Cps2H and LgtD looked very similar in these regions (data not shown). Based on sequence homology the *lgtD* gene product was suggested to have glycosyl transferase activity (U32768).

The gene product encoded by the *cps2I* gene showed some similarity with a protein of *Actinobacillus actinomycetemcomitans* (AB002668). This protein is part of the gene cluster responsible for the serotype-b-specific antigen of *A. actimycetemcomitans*. The function of the protein is unknown.

The gene products encoded by the *cps2J* and *cps2K* genes showed significant similarities to the Cps14J protein of *S. pneumoniae*. The *cps14J* gene of *S. pneumoniae* was shown to encode a ß-1,4-galactosyltransferase activity. In *S. pneumoniae* CpsJ is responsible for the addition of the fourth (i.e. last) sugar in the synthesis of the *S. pneumoniae* serotype 14 polysaccharide (20). Even some similarity was found between Cps2J and Cps2K (Fig. 2, 25.5% similarity). This similarity was most pronounced in the N-terminal regions of the proteins. Recently, two small conserved regions were identified in the N-terminus of Cps14J and Cps14I and their homologues (20). These regions were predicted to be important for catalytic activity. Both regions, DXS and DXDD (Fig. 2), were also found in Cps2J and Cps2K.

Construction of mutants impaired in capsule production. To evaluate the role of the capsule of *S. suis* type 2 in the pathogenesis, we constructed two isogenic mutants in which capsule production was disturbed. To construct mutant 10cpsB, pCPS11 was used. In this plasmid a part of the *cps2B* gene was replaced by the spectinomycin-resistance gene. To construct mutant strain 10cpsEF the plasmid pCPS28 was used. In pCPS28 the 3'-end of *cps2E* gene as well as the 5'-end of *cps2F* gene were replaced by the spectinomycin-resistance gene. pCPS11 and pCPS28 were used to electrotransform strain 10 of *S. suis* type 2 and spectinomycin-resistant colonies were selected. Southern blotting and hybridization experiments were used to select double cross over integration events (results not shown).
To test whether the capsular structure of the strains 10cpsB and 10cpsEF was disturbed, we used a slide agglutination test using a suspension of the mutant strains in hyperimmune anti-S. suis type 2 serum (44). The results showed that even in the absence of serotype specific antisera, the bacteria agglutinated. This indicates that in the mutant strains the capsular structure was disturbed. To confirm this, thin sections of wild type and mutant strains were compared by electron microscopy. The results showed that compared to the wild type (Fig. 3A) the amount of capsule produced by the mutant strains was greatly reduced (Figs. 3B and 3C). Almost no capsular material could be detected on the surface of the mutant strains.

Capsular mutants are sensitive to phagocytosis and killing by porcine alveolar macrophages (PAM). The capsular mutants were tested for their ability to resist phagocytosis by PAM in the presence of porcine SPF serum. The wild type strain 10 seemed to be resistant to phagocytosis under these conditions (Fig. 4A). In contrast, the mutant strains were efficiently ingested by macrophages (Fig. 4A). After 90 min. more than 99.7% (strain 10cpsB) and 99.8% (strain 10cpsEF) of the mutant cells were ingested by the macrophages. Moreover, as shown in Fig. 4B the

ingested strains were efficiently killed by the macrophages. 90-98% of all ingested cells were killed within 90 min. No differences could be observed between wild type and mutant strains. These data indicate that the capsule of *S. suis* type 2 efficiently protects the organism from uptake by macrophages *in vitro*.

Capsular mutants are less virulent for germfree piglets. The virulence properties of the wild-type and mutant strains were tested after experimental infection of newborn germfree pigs (45, 49). Table 1 shows that specific and nonspecific signs of disease could be observed in all pigs inoculated with the wild type strain. Moreover, all pigs inoculated with the wild type strain died during the course of the experiment or were killed because of serious illness or nervous disorders (Table 3). In contrast, the pigs inoculated with strains 10cpsB and 10cpsEF showed no specific signs of disease and all pigs survived until the end of the experiment. The temperature of the pigs inoculated with the wild type strain increased 2 days after inoculation and remained high until day 5 (Table 3). The temperature of the pigs inoculated with the mutant strains sometimes exceeded the 40°C, however, we could observe significant differences in the fever index [i.e % of observations in an experimental group during which pigs showed fever (>40°C)] between pigs inoculated with wild type and mutant strains. All pigs showed increased numbers of polymorphonuclear leucocytes (PMLs) (>10 x 10^9 PMLs per litre) (Table 3). However, in pigs inoculated with the mutant strains the percentage of samples with increased numbers of PMLs was considerably lower. *S. suis* strains and *B. bronchiseptica* could be isolated from the nasopharynx and feces swab samples of all pigs from 1 day post-infection until the end of the experiment (Table 3). Postmortem, the wild type strain could frequently be isolated from the central nervous system (CNS), kidney, heart, liver , spleen, serosae, joints and tonsils. Mutant strains

could easily be recovered form the tonsils, but were never recovered from the kidney, liver or spleen. Interestingly, low numbers of the mutant strains were isolated from the CNS, the serosae, the joints,the lungs and the heart. Taken together, these data strongly indicated that mutant *S. suis* strains, impaired in capsule production, are not virulent for young germfree pigs.

DISCUSSION

We describe the identification and the molecular characterization of the *cps* locus, involved in the capsular polysaccharide biosynthesis, of *S. suis* serotype 2. A region of 16 kb was cloned and sequenced. 14 open reading frames were identified. Most of the genes seemed to belong to a single transcriptional unit, suggesting a co-ordinate control of these genes. Based on sequence similarities we could assign putative functions to most of the gene products. We thereby identified regions involved in regulation (Cps2A), chain length determination (Cps2B, C), export (Cps2C) and biosynthesis (Cps2E, F, G, H, J, K). The overall organization seemed to be similar to that of the *cps* and *eps* gene clusters of a number of Gram-positive bacteria (19, 32, 42). The region involved in biosynthesis is located at the centre of the gene cluster and is flanked by two regions containing genes with more common functions. Although, based on sequence similarities a role of most the gene products in the polysaccharide biosynthesis could be envisaged, the role of the *orf2Z*, *orf2Y* and *orf2X* genes remains unclear sofar. The incomplete *orf2Z* gene was located at the 5'-end of the cloned fragment. Orf2Z showed some similarity with the YitS protein of *B.subtilis*. However, because the function of the YitS protein is unknown this did not give us any information about the possible function of Orf2Z. Because the *orf2Z* gene is not a part of the *cps* operon, a role of this gene in polysaccharide biosynthesis is not expected. The analysis of isogenic mutants impaired in the expression of

Orf2Z should confirm this idea. The Orf2Y protein showed some similarity with the YcxD protein of *B.subtilis* (53).The YcxD protein was suggested to be a regulatory protein. Similarly, Orf2Y may be involved in the regulation of polysaccharide biosynthesis. The Orf2X protein showed similarity with the YAAA proteins of *H. influenzae* and *E. coli*. The function of these proteins is unknown. In *S. suis* type 2 the *orf2X* gene seemed to be the first gene in the *cps2* operon.This suggests a role of Orf2X in the polysaccharide biosynthesis. In *H. influenzae* and *E. coli* , however, these proteins are not associated with capsular gene clusters. The analysis of isogenic mutants impaired in the expression of Orf2X should give more insight in the presumed role of Orf2X in the polysaccharide biosynthesis of *S. suis* type 2.

The gene products encoded by the *cps2E, cps2F, cps2G, cps2H, cps2J* and *cps2K* genes showed some similarity with glycosyltransferases of several Gram-positive or Gram-negative bacteria (18, 19, 20, 22, 25). The *cps2E* gene product showed strong homology with the Cps14E protein of *S. pneumoniae* (18, 19). Cps14E is a glucosyl-1-phosphate transferase that links glucose to a lipid carrier (18). In *S. pneumoniae* this is the first step in the biosynthesis of the oligosaccharide repeating unit. The structure of the *S. suis* serotype 2 capsule is unsolved, but it contains glucose, galactose, rhamnose, N-acetyl glucoseamine and sialic acid in a ratio of 3:1:1:1:1 (7). Based on these data we speculate that Cps2E of *S. suis* could have glucosyltransferase activity, and is probably involved in the linkage of the first sugar to the lipid carrier.

The C-terminal region of the *cps2F* gene product showed some homology with the RfbU of *Salmonella enteritica*. RfbU was shown to have mannosyltransferase activity (24). Because mannosyl is not a component of the *S. suis* type 2 polysaccharide a mannosyltransferase activity is not expected in this organism. Nevertheless, *cps2F* could encode a glycosyltransferase with another sugar specificity.

Cps2G showed moderate homology to a family of gene products suggested to encode galactosyltransferase activities (22, 24, 40). Hence a similar activity can be suggested for Cps2G.

Cps2H showed some similarity with LgtD of *H. influenzae* (U32768). Because LgtD was proposed to have glycosyltransferase activity , a similar activity could be fulfilled by Cps2H.

Cps2J and Cps2K showed homology to Cps14J of *S. pneumoniae* (20). Cps2J showed homology with Cps14I of *S. pneumoniae* as well. Cps14I was shown to have N-acetyl glucosaminyltransferase activity, whereas Cps14J has a ß-1,4-galactosyltransferase activity (20). In *S. pneumoniae* Cps14I is responsible for the addition of the third sugar and Cps14J for the addition of the last sugar in the synthesis of the type 14 repeating unit (20). Because the capsule of *S. suis* type 2 contains galactose as well as N-acetyl glucosamine components, galactosyltransferase as well as N-acetyl glucoaminyltransferase activities could be envisaged for the *cps2J* and *cps2K* gene products, respectively. However, this idea remains to be established by the functional characterization of the Cps2J and Cps2K proteins. As was observed for Cps14I and Cps14J, the N-termini of Cps2J and Cps2K showed a significant degree of sequence similarity. Within the N-terminal domains of Cps14I and Cps14J, two small regions were identified, which were also conserved in several other glycosyltransferases (22). Within these two regions, two Asp residues were proposed to be important for catalytic activity. The two conserved regions, DXS and DXDD, were also found in Cps2J and Cps2K.

The function of Cps2I remains unclear. Cps2I showed some similarity with a protein of *A. actinomycetemcomitans*. Although this protein part is of the gene cluster responsible for the serotype-b-specific antigens, the function of the protein is unknown.

So far, the functions of the *cps2* genes are predicted on the basis of sequence homologies. In future experiments we will concentrate on the functional characterization of the proteins encoded by the various *cps2* genes. Moreover, the analysis of

isogenic mutants in which the individual genes are interrupted, without disturbing expression of the downstream genes, will give more information about the role of the individual *cps2* genes in the polysaccharide biosynthesis of the *S. suis* serotype 2 capsule.

To study the role of the capsule in resistance to phagocytosis and in virulence, we constructed two isogenic mutants in which capsule synthesis was disturbed. In 10cpsB, the *cps2B* gene was disturbed by the insertion of an antibiotic-resistance gene, whereas in 10cpsEF parts of the *cps2E* and *cps2F* genes were replaced. Both mutant strains seemed to be completely unencapsulated. Because the *cps 2* genes seemed to be part of an operon polar effects cannot be excluded. Therefore these data did not give any information about the role of Cps2B, Cps2E or Cps2F in the polysaccharide biosynthesis. However, the results clearly show that the capsular polysaccharide of *S. suis* type 2 is a surface component with antiphagocytic activity. *In vitro* wild type encapsulated bacteria are ingested by phagocytes at a very low frequency, whereas the mutant unencapsulated bacteria are efficiently ingested by porcine macrophages. Within 2 hours, over 99.6% of mutant bacteria were ingested and over 92% of the ingested bacteria were killed. Intracellularly, wild type as well as mutant strains seemed to be killed with the same efficiency. This suggests that the loss of capsular material is associated with loss of capacity to resist uptake by macrophages. This loss of resistance to *in vitro* phagocytosis was associated with a substantial attenuation of the virulence in germfree pigs. All pigs inoculated with the mutant strains survived the experiment and did not show any specific clinical signs of disease. Only some aspecific clinical signs of disease could be observed. Moreover, mutant bacteria could be reisolated from the pigs. This supports the idea that, as in other pathogenic Streptococci, the capsule of *S. suis* acts as an important virulence factor. Recently, a role of the capsule of S. suis type 2 in the pathogenesis was suggested by

Charland et al. (5) as well. Transposon mutants impaired in the capsule production showed a reduced virulence in pigs and mice. To construct these mutants the type 2 reference strain S735 was used. We previously showed that this strain is only weakly virulent for young pigs (48). Moreover, the insertion site of the transposon is unsolved sofar.

TABLE 1. Bacterial strains and plasmids

strain/plasmid	relevant characteristics	source/reference
Strain		
E.coli		
CC118	PhoA$^-$	(28)
XL2 blue		Stratagene
E.coli		
XL2 blue		Stratagene
S. suis		
10	virulent serotype 2 strain	(49)
3	serotype 2	(63)
17	serotype 2	(63)
735	reference strain serotype 2	(63)
T15	serotype 2	(63)
6555	reference strain serotype 1	(63)
6388	serotype 1	(63)
6290	serotype 1	(63)
5637	serotype 1	(63)
5673	serotype 1/2	(63)
5679	serotype 1/2	(63)
5928	serotype 1/2	(63)
5934	serotype 1/2	(63)
5209	reference strains serotype 1/2	(63)
5218	reference strain serotype 9	(63)
5973	serotype 9	(63)
6437	serotype 9	(63)
6207	serotype 9	(63)
reference strains	serotypes 1-34	(9, 56, 14)
S. suis		
10	virulent serotype 2 strain	(51)
10cpsB	isogenic cpsB mutant of strain 10	this work
10cpsEF	isogenic cpsEF mutant of strain 10	this work
Plasmid		
pKUN19	replication functions pUC, Amp^R	(23)
pGEM7Zf(+)	replication functions pUC, Amp^R	Promega Corp.
pIC19R	replication functions pUC, Amp^R	(29)
pIC20R	replication functions pUC, Amp^R	(29)
pIC-spc	pIC19R containing spc^R gene of pDL282	labcollection
pDL282	replication functions of pBR322 and pVT736-1, Amp^R, Spc^R	(43)
pPHOS2	pIC-spc containing the truncated *pho*A gene of pPHO7 as a *Pst*I-*Bam*HI fragment	this work
pPHO7	contains truncated *pho*A gene	(15)
pPHOS7 pPHOS2	containing chromosomal *S. suis* DNA	this work
pCPS6	pKUN19 containing 6 kb *Hin*dIII fragment of *cps* operon	this work (Fig.1)
pCPS7	pKUN19 containing 3,5 kb *Eco*RI-*Hin*dIII fragment of *cps* operon	this work (Fig.1)
pCPS11 pCPS7	in which 0.4 kb *Pst*I-*Bam*HI fragment of *cps*B gene is replaced by Spc^R gene of pIC-spc	this work (Fig.1)
pCPS17 pKUN19	containing 3.1 kb *Kpn*I fragment of *cps* operon	this work (Fig.1)
pCPS18 pKUN19	containing 1.8 kb *Sna*BI fragment of *cps* operon	this work (Fig.1)
pCPS20 pKUN19	containing 3.3 kb *Xba*I-*Hin*dIII fragment of *cps* operon	this work (Fig.1)
pCPS23 pGEM7Zf(+)	containing 1.5 kb *Mlu*I fragment of *cps* operon	this work (Fig.1)
pCPS25 pIC20R	containing 2.5 kb *Kpn*I-*Sal*I fragment of pCPS17	this work (Fig.1)
pCPS26 pKUN19	containing 3.0 kb *Hin*dIII fragment of *cps* operon	this work (Fig.1)
pCPS27 pCPS25	containing 2.3 kb *Xba*I (blunt)-*Cla*I fragment of pCPS20	this work (Fig.1)
pCPS28 pCPS27	containing the 1.2 kb *Pst*I-*Xho*I Spc^R gene of pIC-spc	this work (Fig.1)
pCPS29 pKUN19	containing 2.2 kb *Sac*I-*Pst*I fragment of cps operon	this work (Fig.1)

pCPS1-1 pKUN19	containing 5 kb *EcoRV* fragment of *cps* operon of type 1	this work (Fig.1)
pCPS1-2 pKUN19	containing 2.2 kb *HindIII* fragment of *cps* operon of type 1	this work (Fig.1)
pCPS9-1 pKUN19	containing 1 kb *HindIII-XbaI* fragment of *cps* operon of serotype 9	this work (Fig.1)
pCPS9-2 pKUN19	containing 4.0 kb *XbaI-XbaI* fragment of *cps* operon of serotype 9	this work (Fig.1)

Amp^R: ampicillin resistant
Spc^R: spectinomycin resistant
cps: capsular polysaccharide

TABLE 2. Properties of ORFs in the *cps* locus of *S. suis* serotype 2 and silimarities to gene products of other bacteria

ORF	nucleotide position in sequence	G + C%	number of amino acids	predicted mol. mass (kDa)	predicted pI	proposed function of gene product[1]	Similar gene product (% identity)	reference
ORF Z	? -719		?				*Bacillus subtilis* YitS (26%)	(Y09478)
ORF Y	2079-822	37.9	419	49.4	8.0		*Bacillus subtilis YcxD* (39%)	(53)
ORF X	2202-2934	38.5	244	28.4	8.1		*Haemophilus influenzae* YAAA (24%)	(P43908)
							Escherichia coli YAAA (21%)	(P11288)
Cps2A	3041-4484	38.7	481	53.3	7.9	Regulation	*Streptococcus pneumoniae* Cps19fA (58%)	(12, 29)
							Streptococcus pneumoniae Cps14A (57%)	(19)
							Streptococcus pneumoniae Cap1A (57%)	(30)
							Streptococcus thermophilus EpsA (50%)	(40)
							Streptococcus salvarius CpsA,[c] (56%)	(X94980)
Cps2B	4504-5191	40.1	229	25.2	7.6	Chain length determination	*Streptococcus pneumoniae* type 3 Orf1 (58%)	(2)
							Streptococus pneumoniae Cap1C (58%)	(30)
							Streptococcus pneumoniae Cps14C (58%)	(19)
							Streptococcus pneumoniae Cps19fC (58%)	(12, 29)
							Streptococcus thermophilus EpsC (54%)	(40)
							Streptococcus salvarius CpsC (54%)	(X94980)
							Streptococcus agalactiae CpsB (44%)	(34)
Cps2C	5203-5878	40.2	225	24.4	8.0	Chain length determination/ Export	*Streptococcus pneumoniae* Cps19fD (60%)	(12, 29)
							Streptococcus pneumoniae Cps14D (59%)	(19)
							Streptococcus pneumoniae Cap1D (60%)	(30)
							Streptococcus agalactiae CpsC (53%)	(34)
							Streptococcus salvarius CpsD (52%)	(X94980)
							Streptococcus thermophilus EpsD (51%)	(40)
							Lactococcus lactis EpsB (37%)	(42)
Cps2D	5919-6648	38.0	243	28.2	8.0	Unknown	*Streptococcus pneumoniae* Cps19fB (59%)	(12, 29)
							Streptococcus agalactiae CpsA (58%)	(34)
							Streptococcus salvarius CpsB (58%)	(X94980)
							Streptococcus thermophilus EpsB (58%)	(40)
							Streptococcus pneumoniae Cps14B (57%)	(19)

Cps2E	6675-8052	33.4	459	52.9	8.0	Glucosyltransferase	*Streptococcus pneumoniae* Cps14E (56%) *Streptococcus salvarius* CpsE (56%) *Streptococcus pneumoniae* Cps19fE (55%) *Streptococcus agalactiae* CpsD (48%)	(18, 19) (X94980) (29) (34)
Cps2F	8089-9256	32.4	389	45.5	7.8	Glycosyltransferase	*Salmonella enteritica RfbU* (25%)	(25)
Cps2G	9262-10417	35.9	385	43.6	7.9	Glycosyltransferase	*Campylobacter hyoilei* RfbF (25%) *Streptococcus thermophilus* EpsF (25%) *Staphylococcus aureus* Cap1M,[C] (25%) *Streptococcus thermophilus* EpsG (23%)	(22) (40) (24) (40)
Cps2H	10808-12176	31.0	457	53.3	7.9	Glycosyltransferase	*Haemophilus influenzae* LgtD,[N] (28%)	(U32768)
Cps2I	12213-13443	28.8	410	46.9	8.9	Glycosyltransferase	*Actinobacillus actinomycetemcomitans* (28%)	(AB002668)
Cps2J	13583-14579	28.9	332	38.8	7.7	Glycosyltransferase	*Streptococcus pneumoniae* Cps14J (31%) *Streptococcus pneumoniae* Cps14I (27%) *Streptococcus thermophilus* EpsI (29%) *Lactococcus lactis* EpsG,[N] (39%)	(20) (20) (40) (42)
Cps2K	14574-?		?			Glycosyltransferase	*Streptococcus pneumoniae* Cps14J (44%) *Streptococcus thermophilus* EpsI (39%) *Lactococcus lactis* EpsG (39%)	(20) (40) (42)

[1]Predicted by sequence similarity
[N] Similarity refers to the amino-terminal part of the gene product
[C] Similarity refers to the carboxy-terminal part of the gene product

TABLE 3. Virulence of wild type and capsular mutant *S. suis* strains in germfree pigs

S. suis strains[1]	pigs/ group [n]	mortality[2] [%]	morbidity[3] [%]	clinical index of the group [%]		fever index[7]	leuco-cyte		isolation of *S.* [n]	
				spec symptoms[5]	non-spec. symptoms[6]				index[8]	
	joints								CNS	serosae
10	4	100	100	11	88	43	44	2	3	4
10cpsB	4	0	0	0	10	1	3	1	3	2
10cpsEF	4	0	0	0	0	1	0	1	3	2

[1] strain10 in the wild type strain, strains 10cpsB and 10cpsEF are isogenic capsular mutant strains

[2] piglets which died spontaneously or had to be killed for animal welfare reasons

[3] only considering pigs with specific symptoms

[4] clinical index: % of observations which matched the described criteria

[5] specific symptoms: ataxia, lemeness on at lest one joint, stiffness

[6] non-specific symptoms: inappetance, depression

[7] % of observations in the experimental group with a body temperature > 40^0 C

[8] % of blood samples in the group in which number of granulocytes > 10^{10}/l

LEGENDS TO FIGURES

Fig.1.

Genetic organization of the Streptococcus *cps* gene cluster.
(A) The arrows represent potential Orfs. Gene designations are indicated below the arrows.
(B) Physical map and genetic organization of the *cps2* locus. Restriction sites are as follows: C: *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; S, *Sna*BI; Sa: *Sac*I; X, *Xba*I.
(C) The DNA fragments cloned in the various plasmids are indicated.

Fig.2.

Alignments of the N-terminal parts of Cps2J and Cps2K. Identical amino acids are marked by bars. The amino acids shown in bold are also conserved in Cps14I, Cps14J of *S. pneumoniae* and several other glycosyltransferases (19). The aspartate residues marked by asterics are strongly conserved.

Fig.3.

Transmission electron micrographs of thin sections of various *S. suis* strains.
(A) wild type strain 10;
(B) mutant strain 10cpsB;
(C) mutant strain 10cpsEF.
Bar = 100 nm

Fig. 4.

(A) Kinetics of phagocytosis of wild type and mutant *S. suis* strains by porcine alveolair macrophages. Phagocytosis was determined as described in Materials and Methods. The Y-axis

represents the number of CFU per milliliter in the supernatant fluids as determined by plate counting, the X-axis represents time in minutes.

□ wild type strain 10;

o mutant strain 10cpsB;

Δ mutant strain 10cpsEF.

(B) Kinetics of intracellular killing of wild type and mutant *S. suis* strains by porcine AM. The intracellular killing was determined as described in Material and Methods. The Y-axis represents the number of CFU per ml in the supernatant fluids after lysis of the macrophages as determined by plate counting, the X-axis represents time in minutes.

□ wild type strain 10;

o mutant strain 10cpsB;

Δ mutant strain 10cpsEF.

Fig. 5

CPS2 nucleotide sequence.

Fig. 6

Amino acid sequences ORFZ,ORFY, ORFX, CPS2A, CPS2B, CPS2C, CPS2D, CPS2E, CPS2F, CPS2G, CPS2H, CPS2I, CPS2J and CPS2K.

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CLAIMS

1. A vaccine comprising a *Streptococcus* mutant deficient in capsular expression.

2. A vaccine according to claim 1 wherein said *Streptococcus* mutant has been derived by recombinant techniques, preferably through homologous recombination.

3. A vaccine according to claim 1 or 2 wherein said *Streptococcus* mutant is derived from a *Streptococcus* group A, *Streptococcus* group B, *Streptococcus suis* or *Streptococcus pneumonia*.

4. A vaccine according to claim 1 to 3 wherein said mutant is capable of surviving in an immune-competent host.

5. A vaccine according to claim 4 wherein said mutant is capable of surviving at least 4-5 days, preferably at least 8-10 days, in said host.

6. A vaccine according to any of claims 1 to 5 comprising a mutant capable of expressing a *Streptococcus* virulence factor or antigenic determinant.

7. A vaccine according to any of claims 1 to 6 comprising a mutant capable of expressing a non-*Streptococcus* protein.

8. A vaccine according to claim 7 wherein said non-Streptococcus protein has been derived from a pathogen.

9. A method for controlling or eradicating a Streptococcal disease in a population comprising vaccinating subjects in said population with a vaccine according to anyone of claims 1 to 8.

10. A method for controlling or eradicating a Streptococcal disease comprising testing a sample collected from at least one subject in a population partly or wholly vaccinated with a vaccine according to anyone of claims 1 to 8 for the presence of encapsulated Streptococcal strains.

11. A method for controlling or eradicating a Streptococcal disease comprising testing a sample collected from at least

one subject in a population partly or wholly vaccinated with a vaccine according to anyone of claims 1 to 8 for the presence of capsule-specific antibodies directed against Streptococcal strains.

12. A method for controlling or eradicating a Streptococcal disease in a population comprising vaccinating subjects in said population with a vaccine according to anyone of claims 1 to 8 and testing a sample collected from at least one subject in said population for the presence of encapsulated Streptococcal strains and/or for the presence of capsule-specific antibodies directed against Streptococcal strains.

13. A method according to any of claims 10 to 12 wherein said Streptococcal disease is caused by *Streptococcus suis*.

ABSTRACT

The invention relates to the field of bacterial vaccines, more in particular to vaccines directed against *Streptococcus* infections. The invention provides use of a *Streptococcus* mutant deficient in capsular expression in a vaccine and a *Streptococcus* vaccine strain that has been derived from a *Streptococcus* mutant deficient in capsular expression, preferably wherein said *Streptococcus* mutant is selected from the group composed of *Streptococcus* group A, *Streptococcus* group B, *Streptococcus suis* and *Streptococcus pneumonia*. The invention further provides a method for controlling or eradicating a Streptococcal disease in a population comprising vaccinating subjects in said population with a vaccine according to the invention and testing a sample collected from at least one subject in said population for the presence of encapsulated Streptococcal strains and/or for the presence of capsule-specific antibodies directed against Streptococcal strains.

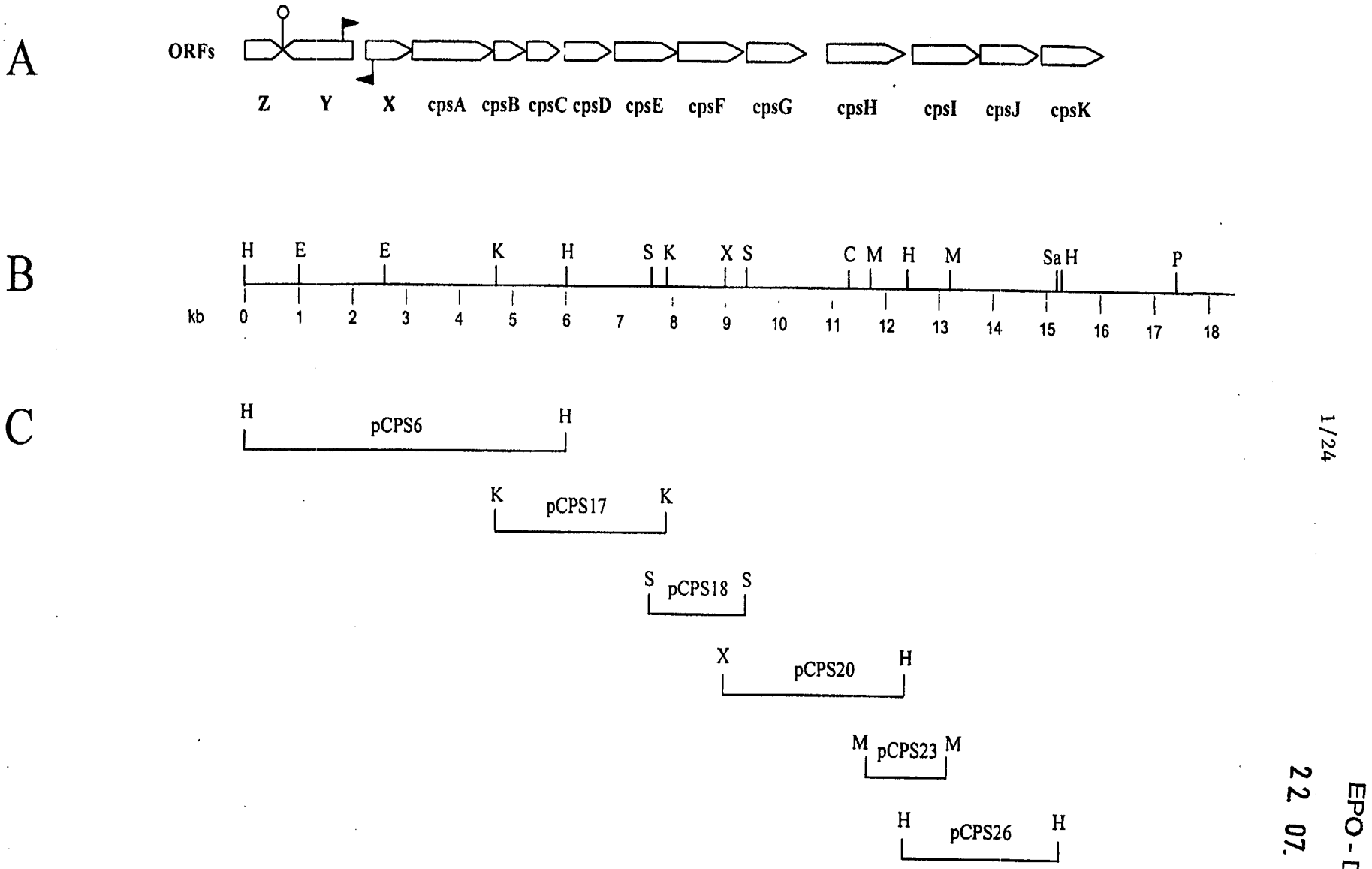

Fig. 1

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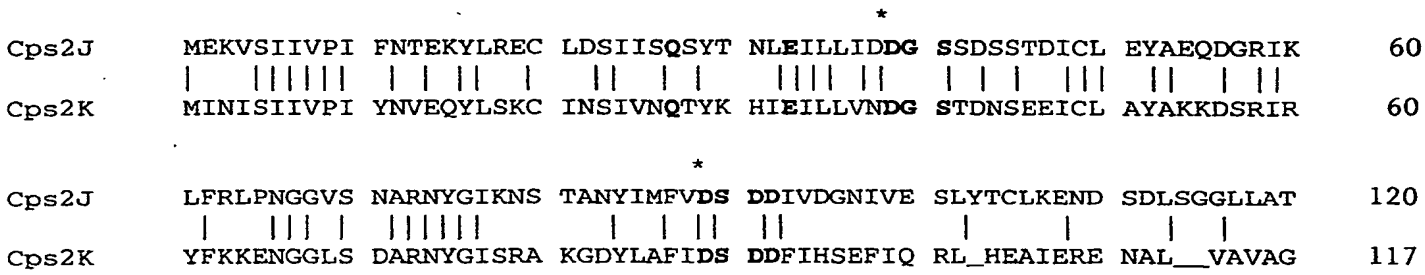
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Cps2J LFRLPNGGVS NARNYGIKNS TANYIMFVDS DDIVDGNIVE SLYTCLKEND SDLSGGLLAT 120
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Fig. 2

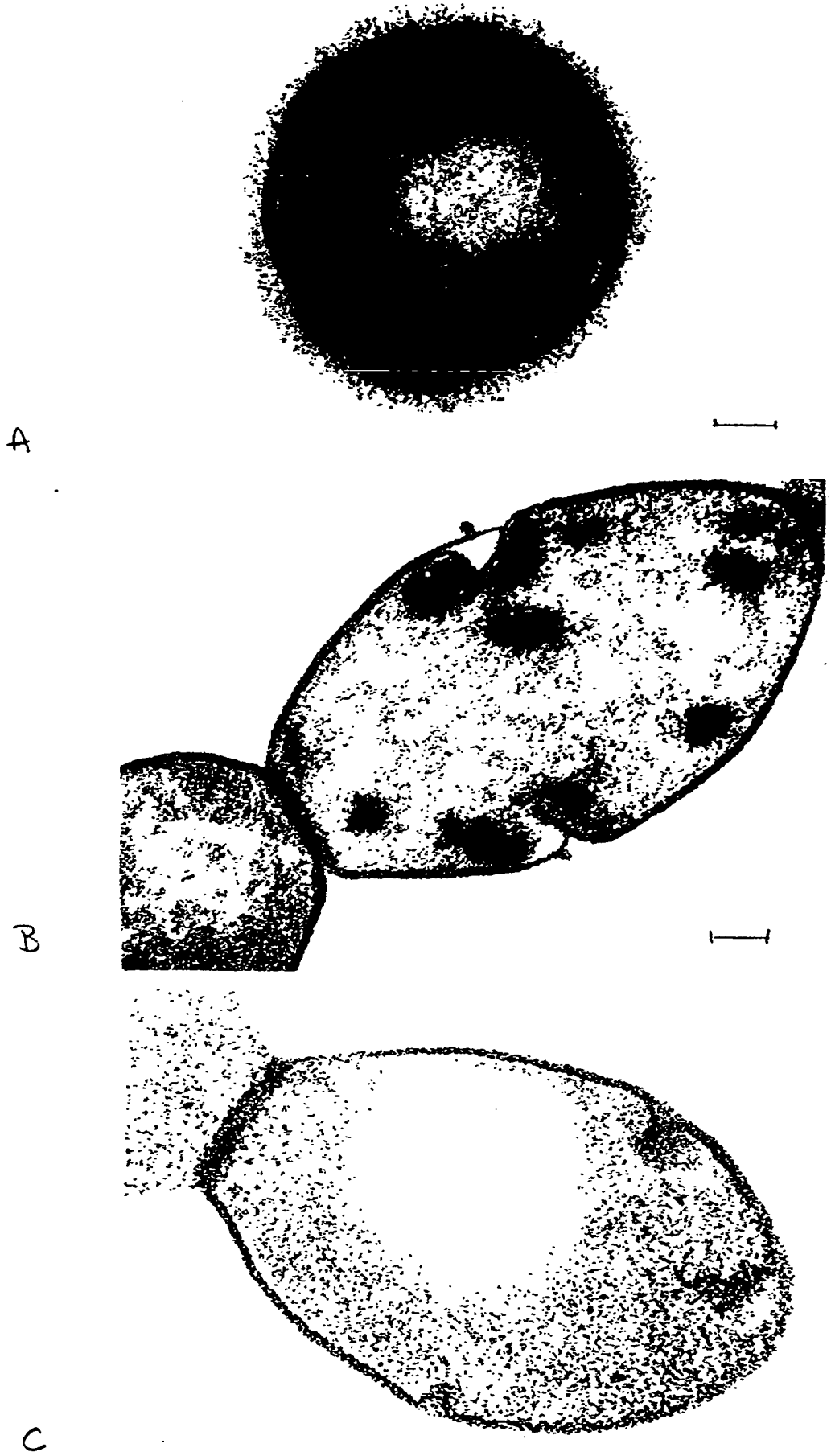

Fig. 3

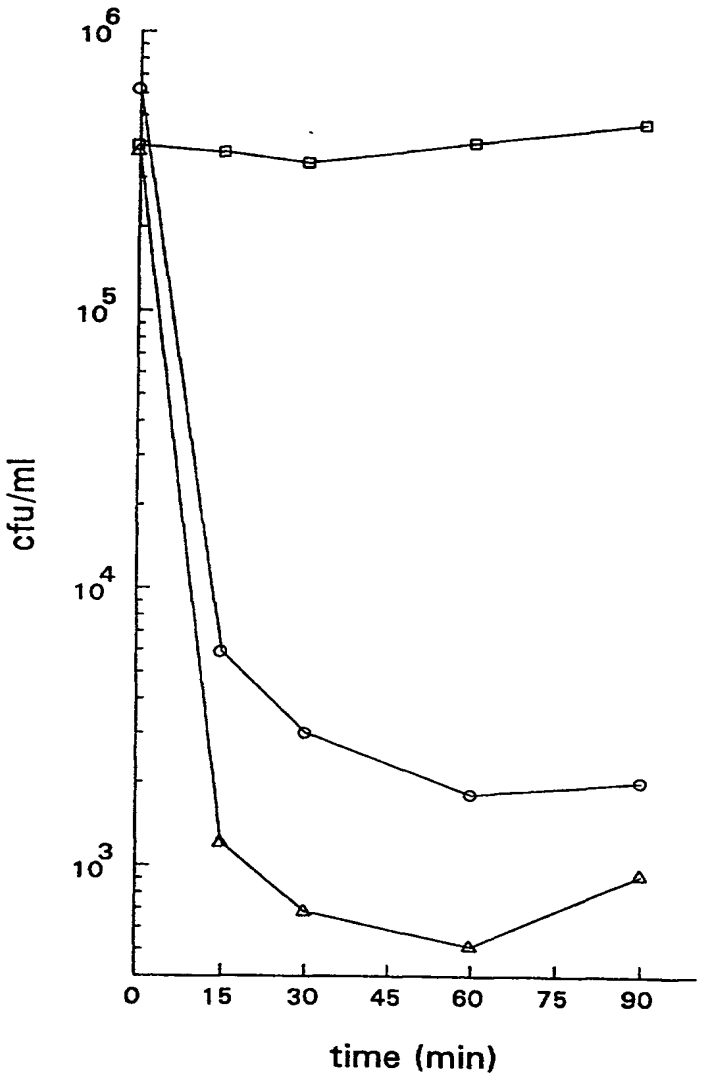

Fig. 4A

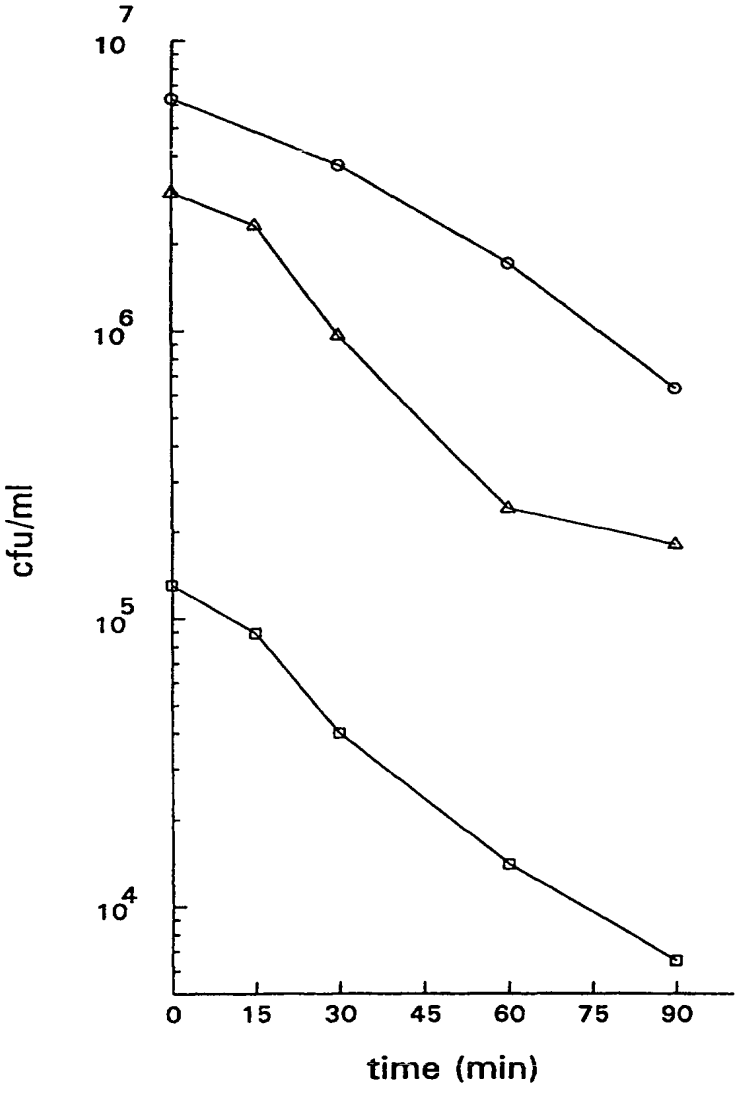

Fig. 4B

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TAGATTTTCC ACAAGTAGTA GAAGCGATAA CTCACTATCG
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Fig. 5

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Fig. 5 cont.

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Fig. 5 cont.

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Fig. 5 cont.

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TGCAAAAGTG TCAAATTGAT TTGGAAGAGA TAAAAGAGGT GCGAGACTTA GGAAATGAAA ATTTTCCCAA
TCATTATATG AGCGGTATCT TTAATAGCCC TTGTTGCAAA
CTTTATAAGA ATATATATAT AAACCAAGGT TTTGACACTG AACAGTGGTT AGGAGAGGAC TTATTATTTA
ATCTAAATTA TTTAAAGAAT ATAAAAAAAG TCCGCTATGT
TAACAGAAAT CTTTATTTTG CCAGAAGAAG TTTACAAAGT ACTACAAATA CGTTTAAATA TGATGTTTTT
ATTCAATTAG AAAATTTAGA AGAAAAAACT TTTGATTTGT
TTGTTAAAAT ATTTGGTGGA CAATATGAAT TTTCTGTTTT TAAAGAGACG CTACAGTGGC ATATTATTTA
TTATAGCTTA TTAATGTTCA AAAATGGAGA TGAATCGCTT
CCAAAGAAAT TGCATATATT TAAGTATTTA TACAATAGGC ATTCTTTAGA TACTCTAAGT ATTAAACGAA
CGTCCTCTGT TTTTAAAAGA ATATGTAAAT TAATTGTTGC
TAATAATTTG TTTAAAATTT TTTTAAATAC TTTAATTAGG GAAGAAAAAA ATAATGATTA ACATTTCTAT
CATCGTCCCA ATTTACAATG TTGAACAATA TCTATCCAAG
TGTATAAATA GCATTGTAAA TCAGACCTAC AAACATATAG AGATTCTTCT GGTGAATGAC GGTAGTACGG
ATAATTCGGA AGAAATTTGT TTAGCATATG CGAAGAAAGA
TAGTCGCATT CGTTATTTTA AAAAAGAGAA CGGCGGGCTA TCAGATGCCC GTAATTATGG CATAAGTCGC
GCCAAGGGTG ACTACTTAGC TTTTATAGAC TCAGATGATT
TTATTCATTC GGAGTTCATC CAACGTTTAC ACGAAGCAAT TGAGAGAGAG AATGCCCTTG TGGCAGTTGC
TGGTTATGAT AGGGTAGATG CTTCGGGGCA TTTCTTAACA
GCAGAGCCGC TTCCTACAAA TCAGGCTGTT CTGAGCGGCA GGAATGTTTG TAAAAAGCTG CTAGAGGCGG
ATGGTCATCG CTTTGTGGTG GCCTGGAATA AACTCTATAA
AAAAGAACTA TTTGAAGATT TTCGATTTGA AAAGGGTAAG ATTCATGAAG ATGAATACTT CACTTATCGC
TTGCTCTATG AGTTAGAAAA AGTTGCAATA GTTAAGGAGT
GCTTGTACTA TTATGTTGAC CGAGAAAATA GTATCATAAC TTCTAGTATG ACTGACCATC GCTTCCATTG
CCTACTGGAA TTTCAAAATG AACGAATGGA CTTCTATGAA
AGTAGAGGAG ATAAAGAGCT CTTACTAGAG TGTTATCGTT CATTTTTAGC CTTTGCTGTT TTGTTTTTAG
GCAAATATAA TCATTGGTTG AGCAAACAGC AAAAGAAGCT
TCTCCAAACG CTATTTAGAA TTGTATATAA ACAATTGAAG CAAAATAAGC GACTTGCTTT ACTAATGAAT
GCTTATTATT TGGTAGGGTG TCTTCATCTT AATTTTAGTG
TCTTTCTGAA AACGGGGAAA GATAAAATTC AAGAAAGATT GAGAAGAAGT GAAAGTAGTA CTCGGTAAGA
ATGTTGTAAT AAATGGTTGA AAGAAAAGGG GATTAAAATG
AATCCAACAA ATAGTAGAAT AGCACTCTTT GATACGATTA AATGTATCAT GGTACTTTGT GTTATTTTTA
CACATCTGGA TTGGTCTGTT GAGCAGCGTC CATGGTTTAT
CTTTCCGTAT TTCGTTGACA TGGCTGTTCC AATTTTCNGT TGCTTCTGCC TATTTTCN

Fig. 5 cont.

ORF Z

SLDIDHMMEVMEASKSAAGSACPSPQAYQAAFEGAENIIVVTITGGLSGSFNAARVARDM
YIEEHPNVNIHLIDSLSASGEMDLLVHQINRLISAGLDFPQVVEAITHYREHSKLLFVLA
KVDNLVKNGRLSKLVGTVVGLLNIRMVGEASAEGKLELLQKARGHKKSVTAAFEEMKKAG
YDGGRIVMAHRNNAKFFQQFSELVKASFPTAVIDEVATSGLCSFYAEEGGLLMGYEVKA

Fig. 6

ORF Y

MKKYQVIIQDILTGIEEHRFKRGEKLPSIRQLREQYHCSKDTVQKAMLELKYQNKIYAVE
KSGYYILEDRDFQDHTCRAQSYRLSRITYEDFRICLKESLIGRENYLFNYYHQQEGLAEL
ISSVQSLLMDYHVYTKKDQLVITAGSQQALYILTQMETLAGKTEILIENPTYSRMIELIR
HQGIPYQTIERNLDGIDLEELESIFQTGKIKFFYTIPRLHNPLGSTYDIATKTAIVKLAK
QYDVYIIEDDYLADFDSSHSLPLHYLDTDNRVIYIKSFTPTLFPALRIGAISLPNQLRDI
FIKHKSLIDYDTNLIMQKALSLYIDNGMFARNTQHLHHIYHAQWNKIKDCLEKYALNIPY
RIPKGSVTFQLSKGILSPSIQHMFGKCYYFSGQKADFLQIFFEQDFADKLEQFVRYLNE

Fig. 6 cont.

ORF X

MKIIIPNAKEVNTNLENASFYLLSDRSKPVLDAISQFDVKKMAAFYKLNEAKAELEADRW
YRIRTGQAKTYPAWQLYDGLMYRYMDRRGIDSKEENYLRDHVRVATALYGLIHPFEFISP
HRLDFQGSLKIGNQSLKQYWRPYYDQEVGDDELILSLASSEFEQVFSPQIQKRLVKILFM
EEKAGQLKVHSTISKKGRGRLLSWLAKNNIQELSDIQDFKVDGFEYCTSESTANQLTFXR
SIKM

Fig. 6 cont.

CPS2A

MKKRSGRSKSSKFKLVNFALLGLYSITLCLFLVTMYRYNILDFRYLNYIVTLLLVGVAVL
AGLLMWRKKARIFTALLLVFSLVITSVGIYGMQEVVKFSTRLNSNSTFSEYEMSILVPAN
SDITDVRQLTSILAPAEYDQDNITALLDDISKMESTQLATSPGTSYLTAYQSMLNGESQA
MVFNGVFTNILENEDPGFSSKVKKIYSFKVTQTVETATKQVSGDSFNIYISGIDAYGPIS
TVSRSDVNIIMTVNRATHKILLTTTPRDSYVAFADGGQNQYDKLTHAGIYGVNASVHTLE
NFYGIDISNYVRLNFISFLQLIDLVGGIDVYNDQEFTSLHGNYHFPVGQVHLNSDQALGF
VRERYSLTGGDNDRGKNQEKVIAALIKKMSTPENLKNYQAILSGLEGSIQTDLSLETIMS
LVNTQLESGTQFTVESQALTGTGRSDLSSYAMPGSQLYMMEINQDSLEQSKAAIQSVLVE
K

Fig. 6 cont.

CPS2B

MNNQEVNAIEIDVLFLLKTIWRKKFLILLTAVLTAGLAFVYSSFLVTPQYDSTTRIYVVS
QNVEAGAGLTNQELQAGTYLAKDYREIILSQDVLTQVATELNLKESLKEKISVSIPVDTR
IVSISVRDADPNEAARIANSLRTFAVQKVVEVTKVSDVTTLEEAVPAEEPTTPNTKRNIL
LGLLAGGILATGLVLVMEVLDDRVKRPQDIEEVMGLTLLGIVPDSKKLK

Fig. 6 cont.

CPS2C

MAMLEIARTKREGVNKTEEYFNAIRTNIQLSGADIKVVGITSVKSNEGKSTTAASLAIAY
ARSGYKTVLVDADIRNSVMPGFFKPITKITGLTDYLAGTTDLSQGLCDTDIPNLTVIESG
KVSPNPTALLQSKNFENLLATLRRYYDYVIVDCPPLGLVIDAAIIAQKCDAMVAVVEAGN
VKCSSLKKVKEQLEQTGTPFLGVILNKYDIATEKYSEYGNYGKKA

Fig. 6 cont.

CPS2D

MIDIHSHIIFGVDDGPKTIEESLSLISEAYRQGVRYIVATSHRRKGMFETPEKIIMINFL
QLKEAVAEVYPEIRLCYGAELYYSKDILSKLEKKKVPTLNGSCYILLEFSTDTPWKEIQE
AVNEMTLLGLTPVLAHIERYDALAFQSERVEKLIDKGCYTQVNSNHVLKPALIGERAKEF
KKRTRYFLEQDLVHCVASDMHNLYSRPPFMREAYQLVKKEYGEDRAKALFKKNPLLILKN
QVQ

Fig. 6 cont.

CPS2E

MNIEIGYRQTKLALFDMIAVTISAILTSHIPNADLNRSGIFIIMMVHYFAFFISRMPVEF
EYRGNLIEFEKTFNYSIIFVIFLMAVSFMLENNFALSRRGAVYFTLINFVLVYLFNVIIK
QFKDSFLFSTTYQKKTILITTAELWENMQVLFESDILFQKNLVALVILGTEIDKINLPLP
LYYSVEEAIGFSTREVVDYVFINLPSEYFDLKQLVSDFELLGIDVGVDINSFGFTVLKNK
KIQMLGDHSIVTFSTNFYKPSHIWMKRLLDILGAVVGLIISGIVSILLIPIIRRDGGPAI
FAQKRVGQNGRIFTFYKFRSMFVDAEVRKKELMAQNQMQGGMFKMDNDPRITPIGHFIRK
TSLDELPQFYNVLIGDMSLVGTRPPTVDEFEKYTPSQKRRLSFKPGITGLWQVSGRSDIT
DFNEVVRLDLTYIDNWTIWSDIKILLKTVKVVLLREGGQ

Fig. 6 cont.

CPS2F

MRTVYIIGSKGIPAKYGGFETFVEKLTEYQKDKSINYFVACTRENSAKSDITGEVFEHNG
ATCFNIDVPNIGSAKAILYDIMALKKSIEIAKDRNDTSPIFYILACRIGPFIYLFKKQIE
SIGGQLFVNPDGHEWLREKWSYPVRQYWKFSESLMLKYADLLICDSKNIEKYIHEDYRKY
APETSYIAYGTDLDKSRLSPTDSVVREWYKEKEISENDYYLVVGRFVPENNYEVMIREFM
KSYSRKDFVLITNVEHNSFYEKLKKETGFDKDKRIKFVGTVYNQELLKYIRENAFAYFHG
HEVGGTNPSLLEALSSTKLNLLLDVGFNREVGEEGAKYWNKDNLHRVIDSCEQLSQEQIN
DMDSLSTKQVKERFSWDFIVDEYEKLFKG

Fig. 6 cont.

CPS2G

MKKILYLHAGAELYGADKVLLELIKGLDKNEFEAHVILPNDGVLVPALREVGAQVEVINY
PILRRKYFNPKGIFDYFISYHHYSKQIAQYAIENKVDIIHNNTTAVLEGIYLKRKLKLPL
LWHVHEIIVKPKFISDSINFLMGRFADKIVTVSQAVANHIKQSPHIKDDQISVIYNGVDN
KVFYQSDARSVRERFDIDEEALVIGMVGRVNAWKGQGDFLEAVAPILEQNPKAIAFIAGS
AFEGEEWRVVELEKKISQLKVSSQVXRMDYYANTTELYNMFDIFVLPSTNPDPLPTVVLK
AMACGKPVVGYRHGGVCEMVKEGVNGFLVTPNSPLNLSKVILQLSENINLRKKIGNNSIE
RQKEHFSLKSYVKNFSKVYTSLKVY

Fig. 6 cont.

CPS2H

MKIISFTMVNNESEIIESFIRYNYNFIDEMVIIDNGCTDNTMQIIFNLIKEGYKISVYDE
SLEAYNQYRLDNKYLTKIIAEKNPDLIIPLDADEFLTADSNPRKLLEQLDLEKIHYVNWQ
WFVMTKKDDINDSFIPRRMQYCFEKPVWHHSDGKPVTKCIISAKYYKKMNLKLSMGHHTV
FGNPNVRIEHHNDLKFAHYRAISQEQLIYKTICYTIRDIATMENNIETAQRTNQMALIES
GVDMWETAREASYSGYDCNVIHAPIDLSFCKENIVIKYNELSRETVAERVMKTGREMAVR
AYNVERKQKEKKFLKPIIFVLDGLKGDEYIHPNPSNHLTILTEMYNVRGLLTDNHQIKFL
KVNYRLIITPDFAKFLPHEFIVVPDTXDIEQVKSQYVGTGVDLSKIISLKEYRKEIGFIG
NLYALLGFVPNMLNRIYLYIQRNGIANTIIKIKSRL.

Fig. 6 cont.

CPS2I

MQADRRKTFGKMRIRINNLFFVAIAFMGIIISNSQVVLAIGKASVIQYLSYLVLILCIVN
DLLKNNKHIVVYKLGYLFLIIFLFTIGICQQILPITTKIYLSISMMIISVLATLPISLIK
DIDDFRRISNHLLFALFITSILGIKMGATMFTGAVEGIGFSQGFNGGLTHKNFFGITILM
GFVLTYLAYKYGSYKRTDRFILGLELFLILISNTRSVYLILLLFLFLVNLDKIKIEQRQW
STLKYISMLFCAIFLYYFFGFLITHSDSYAHRVNGLINFFEYYRNDWFHLMFGAADLAYG
DLTLDYAIRVRRVLGWNGTLEMPLLSIMLKNGFIGLVGYGIVLYKLYRNVRILKTDNIKT
IGKSVFIIVVLSATVENYIVNLSFVFMPICFCLLNSISTMESTINKQLQT

Fig. 6 cont.

CPS2J

MEKVSIIVPIFNTEKYLRECLDSIISQSYTNLEILLIDDGSSDSSTDICLEYAEQDGRIK
LFRLPNGGVSNARNYGIKNSTANYIMFVDSDDIVDGNIVESLYTCLKENDSDLSGGLLAT
FDGNYQESELQKCQIDLEEIKEVRDLGNENFPNHYMSGIFNSPCCKLYKNIYINQGFDTE
QWLGEDLLFNLNYLKNIKKVRYVNRNLYFARRSLQSTTNTFKYDVFIQLENLEEKTFDLF
VKIFGGQYEFSVFKETLQWHIIYYSLLMFKNGDESLPKKLHIFKYLYNRHSLDTLSIKRT
SSVFKRICKLIVANNLFKIFLNTLIREEKNND

Fig. 6 cont.

CPS2K

MINISIIVPIYNVEQYLSKCINSIVNQTYKHIEILLVNDGSTDNSEEICLAYAKKDSRIR
YFKKENGGLSDARNYGISRAKGDYLAFIDSDDFIHSEFIQRLHEAIERENALVAVAGYDR
VDASGHFLTAEPLPTNQAVLSGRNVCKKLLEADGHRFVVAWNKLYKKELFEDFRFEKGKI
HEDEYFTYRLLYELEKVAIVKECLYYYVDRENSIITSSMTDHRFHCLLEFQNERMDFYES
RGDKELLLECYRSFLAFAVLFLGKYNHWLSKQQKKLLQTLFRIVYKQLKQNKRLALLMNA
YYLVGCLHLNFSVFLKTGKDKIQERLRRSESSTR.

Fig. 6 cont.